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Quinolone Resistance in *Neisseria gonorrhoeae*: Rapid Genotyping of Quinolone Resistance-Determining Regions in *gyrA* and *parC* Genes by Melting Curve Analysis Predicts Susceptibility▽

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We report a duplex real-time PCR assay for the simultaneous screening of mutations involved in fluoroquinolone resistance within *gyrA* and *parC* quninolone resistance-determining regions (QRDRs) in *Neisseria gonorrhoeae*. Our assay clearly detects all mutated QRDRs and allows the identification of common genotypes, whether the QRDRs contain single or double mutations, providing valuable epidemiological tools. When this method is used in conjunction with similar assays and in vitro analyses, essential antibiotic resistance surveillance can be performed for public health purposes.

Gonorrhea rates generally remain high in New Caledonia and Oceania, where penicillins are the recommended treatment (17, 19, 22). Increasing penicillin resistance means that fluoroquinolones are the next therapeutic option, but monitoring of relevant antibiotics for effectiveness for public health purposes (2, 7, 15, 16, 23) is required. However, resistance surveillance of gonococci is difficult because of culture-related and storage issues (3). Nonetheless, important epidemiological data may now be obtained if appropriate samples are conserved for genetic analysis (22).

The main determinant of fluoroquinolone resistance in gonococci is target site alteration by spontaneous mutations in the quinolone resistance-determining regions (QRDRs) of *gyrA* and *parC* (1, 4, 6, 20). The codons 91 and 95 of *gyrA* and 86 through 88 and 91 of *parC* are those most frequently associated with quinolone resistance in *Neisseria gonorrhoeae* (1, 6, 11, 13, 14, 20). The stepwise acquisition of mutations leading to progressive increases in MICs begins first with *gyrA* changes resulting in a less susceptible or resistant phenotype, followed by *parC* alterations that increase the resistance. We developed and validated a real-time PCR-based genotyping method using hybridization probes to detect QRDR mutations within *gyrA* and *parC* for the epidemiological surveillance of quinolone susceptibility.

The QRDR sequences and ciprofloxacin MICs for a panel of 21 selected *N. gonorrhoeae* WHO reference strains and other strains from the WHO Collaborating Centre in Sydney, Australia, were first determined (Table 1). Strains were categorized as less susceptible when MICs were 0.06 to 0.5 μg/ml, as resistant when MICs were >0.5 to <4 μg/ml, and as highly resistant when MICs were ≥4 μg/ml (18). A further 14 clinical strains isolated at the Institut Pasteur in Cambodia, for which MICs of ciprofloxacin ranged from 0.016 to >32 μg/ml (Table 1), and a 1-year collection of 100 isolates (only 1 of which was quinolone-resistant *N. gonorrhoeae* [QRNG]) from the bacteriology laboratory of the Institut Pasteur in New Caledonia were also examined. The ciprofloxacin MICs for these isolates were determined by the Etest (AB Biodisk, Solna, Sweden) diffusion method using WHO reference strains as controls. All strains and isolates were handled and DNA was extracted as described previously (22). Oligonucleotides were designed using the LightCycler probe design software version 2.0 (Roche Diagnostics) and synthesized by Sigma-Proligo (Singapore Pty. Ltd). The 761-bp amplified region of *gyrA* was probed for mutations in codons 91 through 95 by melting curve analysis with a LightCycler Red 705 probe. For *parC* analysis, an anchor probe was combined with a LightCycler Red 670-labeled sensor probe overlapping codons 86 through 91 inside a 607-bp amplified *parC* product. Primer and probe sequences and the reaction conditions are summarized in Table 2. Amplification and melting curve analysis of the two genes were conducted in a single multiplex run with a final volume of 20 μl. This protocol ensures multiplex genotyping using a compensation color program to correct emission spectrum overlaps of the dyes. Controls in every experiment included a blank capillary tube, wild-type DNA, and mutated DNA.

We obtained highly reproducible results both within and between successive runs, with distinguishable melting curves for both QRDRs (Fig. 1). The *gyrA* QRDR genotyping analysis used the 705-nm wavelength and identified eight different melting peaks, based on melting temperatures (T_ms) and curve shapes (Fig. 1A). Wild-type *gyrA* QRDR sequences were identified by a mean T_m ± a standard deviation of 57.2 ± 0.1°C. Single mutants were easily evidenced by T_ms in the range of 50.5 to 51.5°C. Among single mutants, D95N and D95G were distinguished by T_ms of 50.7 ± 0.05°C and 51.0 ± 0.1°C, respectively, both with a sharp melting peak, whereas S91Y, S91F, and D95G were not visibly distinguished from one another due to the overlap of their melting peaks, with a T_m of 51.4 ± 0.1°C.

Four profiles specific to double mutants were clearly identified and confirmed by sequencing reactions (Table 1). In descending

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TABLE 1. Reference and collection strains used in this study

Strain[a]	Ciprofloxacin phenotype[b]	Ciprofloxacin MIC (μg/ml)	*gyrA* QRDR genotype[c]	*parC* QRDR genotype	Source
Reference strains					
WHO. P	S	0.016	**wt**	**wt**	
WHO. O	S	0.016	**wt**	**wt**	
WHO. C	S	0.016	wt*	wt*	
07 QA 02	S	0.023	**wt**	**wt**	
93G0065	LS	0.125	D95N*	**wt**	
97G0319	LS	0.125	D95G*	**wt**	
91G0622	LS	0.125	D95N*	**wt**	
WHO. G	LS	0.25	S91F*	**wt**	
98G0959	LS	0.25	D95N*	**wt**	
07 QA 06	LS	0.38	S91F*	**wt**	
91G0316	LS	0.5	S91F*	**wt**	
92G0551	LS	0.5	S91Y*	**wt**	
98G0798	R	1.0	S91F*	**wt**	
96G0142	R	1.0	S91Y*	E91Q*	
97G0398	R	2.0	S91F + D95G*	S88P*	
97G0109	R	2.0	S91F + D95G*	D86N*	
WHO. M	R	2.0	**S91F + D95G**	**wt**	
98G0867	HR	8.0	S91F + D95G*	S87R*	
98G0872	HR	8.0	S91F + D95N*	E91K*	
WHO. L	HR	16	**S91F + D95N**	D86N + S88P*	
95G0142	HR	32	S91F + D95N*	S87R*	
Clinical isolates					
IPC-NG8	S	0.016	wt*	**wt**	Cambodia
IPC-NG16	R	1.5	**S91F + D95A**	S87R*	Cambodia
IPC-NG3	R	3.0	S91F + D95A*	**wt**	Cambodia
IPC-NG14	R	3.0	S91F + D95A*	**wt**	Cambodia
108 175 340	R	4.0	**S91F + D95G**	**E91G**	New Caledonia (import)
IPC-NG13	HR	12	S91F + D95A*	S87N + E91Q*	Cambodia
IPC-NG9	HR	16	**S91F + D95A**	S87N + E91Q*	Cambodia
IPC-NG1	HR	>32	S91F + D95Y*	E91G*	Cambodia
IPC-NG2	HR	>32	S91F + D95Y*	E91G*	Cambodia
IPC-NG5	HR	>32	S91F + D95A*	E91G*	Cambodia
IPC-NG6	HR	>32	S91F + D95Y*	E91G*	Cambodia
IPC-NG7	HR	>32	S91F + D95G*	S87N + E91Q*	Cambodia
IPC-NG10	HR	>32	S91F + D95G*	S87R*	Cambodia
IPC-NG18	HR	>32	S91F + D95A*	S87N*	Cambodia
IPC-NG19	HR	>32	S91F + D95G*	S87R*	Cambodia

[a] IPC strains were kindly provided by B. Guillard, Institut Pasteur of Cambodia.
[b] S stands for susceptible (MIC of <0.06 μg/ml), LS for less susceptible (MIC of 0.06 to 0.5 μg/ml), R for resistant (MIC of >0.5 to <4 μg/ml), and HR for highly resistant (MIC of ≥4 μg/ml).
[c] wt, wild type. Asterisks indicate QRDR genotypes confirmed by sequencing either at the University of New South Wales facility (Sydney, Australia) for *gyrA* or at Waikato University in Hamilton (New Zealand) for *parC*. Boldface indicates genotypes identified based on melting peaks (not sequenced during this study).

TABLE 2. Primers, probes, and amplification conditions[a] used to genotype QRDRs of *N. gonorrhoeae gyrA* and *parC* genes

Oligonucleotide	5′→3′ nucleotide sequence[b]	Position[c]
Primers		
NG-gyrA-F	CGCCACGACCACAAATTC	19–36
NG-gyrA-R	CCTATGGGTTCGATATGGGTCTTA	779–756
NG-parC-F	CGCTTCCCATACCGATTCCAA	18–38
NG-parC-R	TTCCAGCGTCGGTTTCT	624–608
Probes		
NG-gyrA-P1	ATTCCGCCGTTTACGACACC-fluorescein	269–288
NG-gyrA-P2	LC-R705-CGTCCGTATGGCGCAAAATT TCGCTATGCGT-phosphate	291–321
NG-parC-P3	AAGGTAAAATCCTGAGCCA TGCGCACCAT-fluorescein	305–277
NG-parC-P4	LC-R670-CCTCATAGGCGG AACTGTCGC-phosphate	274–254

[a] Single PCR mixtures contained 4 mM $MgCl_2$, and PCRs were run for 50 cycles of 95°C for 8 s, 55°C for 8 s, and 72°C for 30 s. Melting curve conditions were 95°C for 5 s and 37°C for 15 s, with a ramp to 80°C at 0.1°C/s with continuous fluorescence acquisition.
[b] LC-R705, LightCycler Red 705; LC-R670, LightCycler Red 670.
[c] Positions according to sequences with GenBank accession numbers U08817 (*gyrA* gene) and U08907 (*parC* gene).

order of T_ms, our assay discriminated the S91F/D95A genotype, displaying a T_m of 49.05 ± 0.1°C; the S91F/D95G genotype, with a T_m of 44.0 ± 0.2°C; then the S91F/D95N genotype, with a T_m of 43.35 ± 0.1°C; and lastly, the S91F/D95Y genotype (T_m of 42.35 ± 0.05°C). Furthermore, the S91F/D95N profile was characterized by a melting peak showing a reproducibly high level of fluorescence compared with the S91F/D95G profile, which always displayed a relatively low and oblate melting peak.

By using the 670-nm wavelength, the *parC* QRDR genotyping analysis identified 10 different melting peaks, based on T_ms and curve shapes (Fig. 1B). Wild-type *parC* QRDR sequences displayed a T_m of 68.2 ± 0.1°C. Single mutants were evidenced by T_ms in the range of 59.5 to 65.6°C. Some of the single mutants were clearly identified: those with the mutations E91G (T_m of 65.05 ± 0.05°C) and S87N (T_m of 59.7 ± 0.1°C), as well as those with the mutations S88P (T_m of 62.25± 0.05°C) and E91K (T_m of 60.8 ± 0.05°C). Discrimination between D86N (T_m of 61.6 ± 0.1°C), S87R (T_m of 61.25 ± 0.05°C), and E91Q

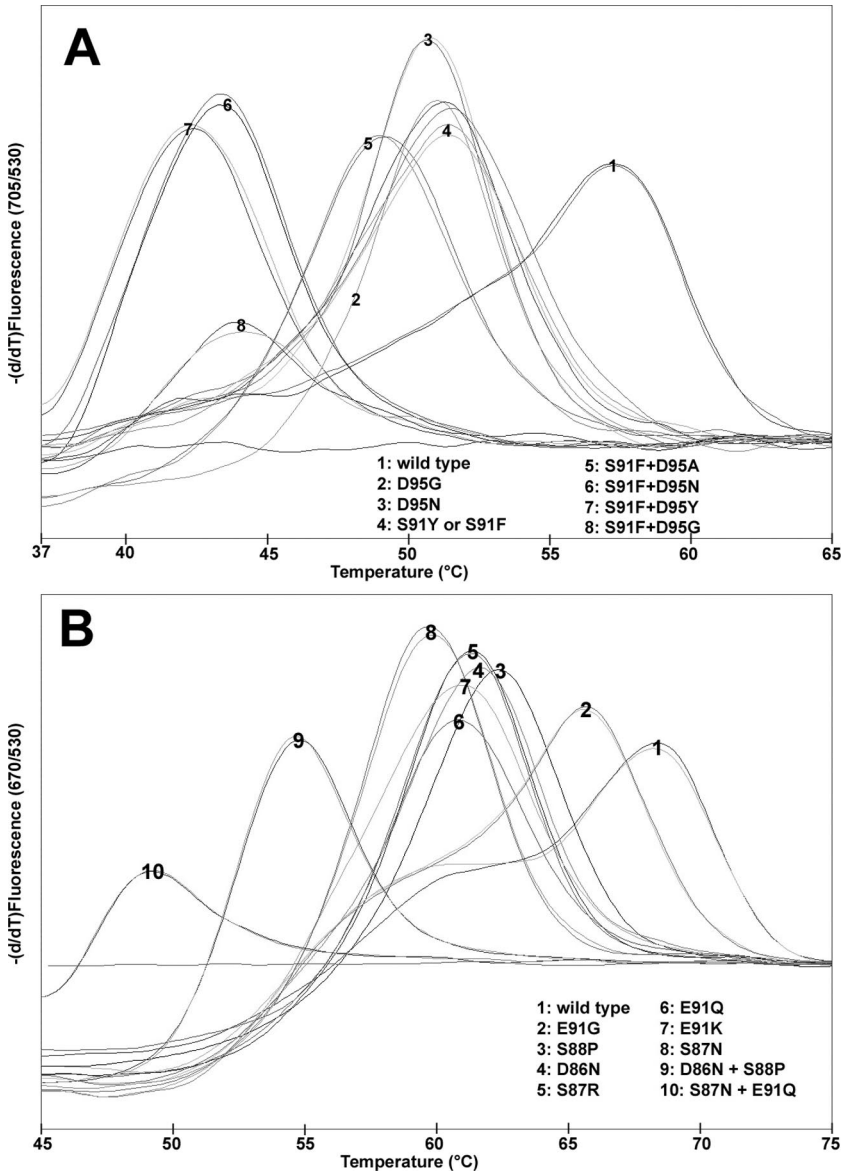

FIG. 1. Representative melting peaks derived from negative derivatives of fluorescence ratios with respect to temperature and corresponding genotypes for *gyrA* (A) and *parC* (B). Both of these spectra were generated using color compensation as indicated in the text.

(T_m of 60.75 ± 0.05°C) was possible thanks to the high level of reproducibility when amplifications of reference DNAs of the corresponding genotypes were included during the same run, allowing a precise comparison of the melting peaks. Double mutants were also identified, with S87N/91Q mutants showing a T_m of 49.15 ± 0.1°C and D86N/S88P mutants showing a T_m of 54.7 ± 0.1°C. A random selection of strains according to their specific melting peaks were sequenced, and all results confirmed the presumptive genotypes assigned based on melting curve analysis.

Accurate genotypic detection of QRNG strains was confirmed by the high correlation with the phenotypic classification of QRNG isolates from Cambodia (where QRNG is highly endemic) and New Caledonia. Phenotypes of decreased susceptibility or moderate resistance correlated with single changes at codon 95 or 91, respectively, in *gyrA*. Further, *parC* alterations were detected only in *gyrA*-mutated strains and corresponded to higher MIC levels than those for strains with *gyrA* changes only, as described previously. No QRNG strains without QRDR changes were detected, and all QRDR alterations were accompanied by phenotypic change. With the inclusion of control DNA representing common genotypes for *gyrA* and *parC* QRDRs, single and double mutations can be easily identified (Fig. 1).

The isolation, storage, and shipment of viable cultures are difficult, expensive, and time-consuming. Nucleic acid amplification techniques may offer complementary means that are useful for diagnosis but also relevant and accurate for the prediction of clinical resistance. Our technique thus allows the provision of important epidemiological data of public health relevance, even if cultures are nonviable (3), through the proper use of a relevant reference center with established

facilities. The detection of resistant subtypes of gonococci by *N. gonorrhoeae* multiantigen sequence typing has been reported previously (8), but this correlative approach is limited by the extensive geographic and temporal variation in gonococcal subtypes. Other strategies to detect QRNG either are time-consuming and require complex equipment (10) or are limited in their applications (5, 12) because they are not able to detect all QRDR mutations. In our assay, all the *gyrA* and *parC* QRDR changes can be determined in a single run with a single capillary tube.

The sensitivity of the assay, in common with those of all sequence-specific techniques, is affected by mutations outside the sequence covered by the sensor probe. Resistance resulting from mutations elsewhere would also remain undetected. However, *gyrB* or *parE* changes do not confer significant fluoroquinolone resistance (6), and Qnr-like proteins have not been detected in gonococci (9).

The early detection of stepwise QRDR mutations in gonococci would not only improve surveillance, but also possibly postpone the emergence of multidrug resistance. If used judiciously in conjunction with the formerly described penicillin resistance determinant assays (21, 22) and existing in vitro methods, the assay described herein would contribute significantly to the essential resistance surveillance required in areas such as Oceania.

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